

Short Article

FORMATION OF CHLAMYDOSPORES IN *Phytophthora parsiana* *in vivo* AND *in vitro* AS SURVIVAL PROPAGULES*

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Abstract

The formation of chlamydospores of *Phytophthora parsiana*, a newly described high temperature tolerant pathogen of woody plants, were investigated in culture media and in infected roots of susceptible host plants. Different solid agar media and cleared V-8 juice broth were inoculated with different isolates of *P. parsiana* and incubated for four days at room temperature before they were transferred to 4°C in the dark. Chlamydospore formation was monitored during twelve months. Infected almond and pistachio roots inoculated with the pathogen under greenhouse conditions were removed from soil, washed thoroughly and incubated within moist sterilized sand in plastic bags at 4°C for twelve months and examined monthly using light microscope. Chlamydospores were observed only on carrot agar two weeks after incubation and after four months in infected pistachio and almond roots. No chlamydospores were produced on other solid media and in CV8 juice during 12 months incubation. The average diameter of chlamydospore in culture and in roots were 29.7 and 32.47µm with wall thickness 1.7 and 2.07 µm, respectively. Frozen culture medium and root containing chlamydospores at - 20°C for 72 hours resumed growth after thawing on *Phytophthora* selective medium. Sterilized non inoculated root segments colonized internally by the pathogen did not resume growth after freezing. The isolates did not produce oospores in single or dual cultures with *P. capsici* mating types. It is concluded that under natural conditions chlamydospore is a survival propagule of the pathogen.

Keywords: *Phytophthora*, Chlamydospore, Oomycetes, Almond, Pistachio, Iran.

See Persian text for figures and tables (Pages ۳۴۳-۳۴۴)

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Introduction

Over 100 species of *Phytophthora* have been identified (Brasier 2008) of which about 70% are homothallic and 30% heterothallic (Gallegly and Hong 2008, Erwin and Rebeiro 1996). The principal survival units of *Phytophthora* species are oospores and chlamydospores. Chlamydospore formation on agar media and infected roots have been reported to be only 23% in homothallic and 7% in heterothallic species of *Phytophthora*. (Erwin and Ribeiro 1996, Gallegly and Hong 2008). Investigation on chlamydospore formation in many *Phytophthora* species especially heterothallic species are scanty. This is partly due to the fact that it is not much used in taxonomy discrimination of the species (Erwin and Ribeiro 1996). The well-known heterothallic species of *Phytophthora* that commonly produce chlamydospores in culture include *P. cinnamomi* (Mircetich and Zentmyer 1967), *P. nicotianae* (Tsao 1971) *P. capsici* (Mchau and Coffey 1995) and *P. palmivora* (Alizadeh and Tsao 1985). *P. parsiana*, a newly described high temperature tolerant species (Mostowfizadeh-Ghalamfarsa *et al.* 2008) which is restricted in host range to woody plants (Hajeberahimi and Banihashemi 2011) does not normally produce chlamydospore in culture (Mostowfizadeh-Ghalamfarsa 2005). The objective of the present study was to investigate chlamydospore formation *in vitro* and *in vivo* by *P. parsiana* isolated from different plant species and geographical regions.

Materials and Methods

Twelve hyphal tip isolates of *P. parsiana* from different hosts and geographical regions were used (Table 1). All isolates were sexually sterile and had not formed oospore mated with A1 and A2 mating types of *P. capsici* with optimum temperature of growth at 30°C and maximum 37-40°C.

From the edge of actively growing colonies of the isolates on corn meal agar (CMA), 6mm blocks of mycelium were transferred to CMA, potato dextrose agar (PDA) water agar (WA), hemp seed agar (HSA) cleared V-8 juice agar (CV8A) and carrot agar (CA) and incubated in the dark at room temperature for 4 days before they were transferred to 4°C for 12 months and checked periodically for chlamydospore formation. For liquid medium, 10 ml of cleared CV-8 juice broth was apportioned in

each 60 ml brown screw cap bottle and autoclaved for 30 minutes and after cooled inoculated with one 6mm block of actively growing hyphae of each isolate and incubated at 25°C for five days before they were transferred to 4°C for 12 months and examined monthly for chlamydospore formation.

One month-old susceptible pistachio and almond seedlings were inoculated in each pot using 50 ml of 4-6 weeks old inoculum in vermiculate hemp seed extract as reported earlier (Banihashemi 2004) and grown under greenhouse conditions. Ten days after seedling mortality, roots removed from pots and washed under running tap water and buried within sterile moist sand and incubated at 4°C for 12 months. Non inoculated roots of both plants were also used as controls. At monthly intervals, roots samples were removed, freed from sand under running tap water, soaked for one hour in 3% KOH to soften the tissues. Root segments were placed on clean microscopic slide and one drop of 1% safranin-o in lactophenol was added and covered with coverslip. The root tissues were squeezed by pressing on the cover slip and observed by light microscope for chlamydospore formation.

To ensure if the chlamydospore is the only propagules in culture and plant roots, the following experiment was designed. Roots of healthy almond and pistachio were autoclaved and placed on the actively growing colonies of the isolate of *P. parsiana* for five days at room temperature. Colonized roots were surface sterilized for 2 minutes in 0.5% sodium hypochlorite, rinsed with sterile distilled water, blotted dry and plated on PARP (Jeffers and Martin 1988) medium to ensure internal infection of the root segment by the pathogen. Culture medium and infected root segments containing chlamydospores and colonized root by mycelium were frozen at -20°C for 72 hours. After thawing the roots and blocks of culture were transferred to PARP medium to observe regrowth of the pathogen.

To observe chlamydospores in intact infected tissues, infected root segments were fixed in formalin aceton ethyl alcohol (FAA) and dehydrated using normal butyl alcohol series embedded in paraffin wax and sectioned using Cambridge rotary microtome (Tuite 1969). The sections were stained in safranin-o, Fast Green (Sass 1958) and observed microscopically. Dimension of chlamydospores observed in all

Table 1. Chlamydospre formation in *Phytophthora parsiana* isolates on carrot agar and in almond roots.

Isolates		Chlamyospore formation		
Code	Source	Location ¹	On carrot agar ²	In almond root ³
PH21.3.08	Pistachio	A	+	+
PH21.5.08	Fig	B	-	+
PH21.6.08	Pistachio	C	+	+
PH21.13.08	Soil	D	-	+
PH21.22.08	Pistachio	C	-	-
PH21.23.10	Pistachio	E	-	-
PH21.24.10	Pistachio	F	-	-
PH21.25.10	Soil	E	-	-
PH21.27.10	Soil	E	+	+
PH21.28.10	Pistachio	G	-	+
PH21.29.10	Pistachio	H	+	+
PH21.31.10	Pistachio	E	-	-

1. Locations : A (California, USA), B (Shabnkareh, Busher Province), C (Rafsanjan, Kerman Province), D (Kerman, Kerman Province), E (Harat, Yazd Provicne), F (Noogh, Rafsanjan, Kerman Province), G (Kabootarkhan, Rafsanjan, Kerman Province), H (Baghin, Kerman, Kerman Province) (B-H= Iran)

+ = Chlamyospore formation

- = No Chlamyospore formed

2. After 15 days at 4°C

3. After 4-6 months at 4°C

preparation was measured using BioLoMICS software version 1.0.2 (BioAware SA NV, 2003).

Results and Discussion

After fifteen days incubation at 4°C, chlamyospores were observed on CA. They were round measuring 29.7µm (14.3-33.1µm) in diameter with average wall thickness 1.4µm (Fig.1). No chlamyospores were formed on other solid or liquid media after 12months incubation. Only 40% of the isolates produced chlamyospore on CA. The formation of chlamyospores in root was delayed until 5-6 months. More than 60% of the isolates produced chlamyospores in pistachio and almond roots. The average dimension of chlamyospore was 32.47 µm (20-44 µm) and 2µm wall thickness (Fig 2). Isolates that were capable of producing chlamyospores in medium also formed in root (Table 1). Thirty percent of the isolates produced chlamyospore in culture and in root tissues although they had been isolated from the same host or location. Infected root and culture

medium containing chlamyospore after freezing at -20°C, resulted in new growth after transferred to PARP. No growth observed on frozen roots colonized by the hyphae.

The role of chlamyospores in the survival of many homothallic and heterothallic species of *Phytophthora* has not been thoroughly investigated. Formation of oospores is important mainly in homothallic species (Zentmyer and Erwin 1970) but their importance in many soil borne heterothallic species is questionable. In airborne *Phytophthora* species like *P. infestance* and *P. ramorum* in the presence of compatible mating types the production of oospore is more probable. Also the frequency of distribution of mating types of soil borne *Phytophthora* species in the region and their ratio is very significant for their production. In some *Phytophthora* species such as *P. parsiana*, *P. irrigata* and *P. hydropathica* only A1 mating type have been reported (Mostowfzadeh-Ghalamfarsa 2005, Hong *et al.* 2008, Hong *et al.* 2010). *P. parsiana* isolates rarely produce oospore by different mating types and considered to be

sterile (Hajebrahimi 2008, Hong *et al.* 2010, Rafiee 2011). Since the morphology of chlamyospores does not vary very considerably among species, its significance for identification is limited (Erwin and Ribeiro 1996). In some instances abundant chlamyospore production has been used as a distinct criteria to delineate the species having similar morphology. *P. parsiana*, *P. irrigata* and *P. hydropathica* have similar morphology and temperature requirement for growth and considered to be high temperature tolerant species. Chlamyospore formation has been reported only in *P. hydropathica* (Hong *et al.* 2010). The optimal environmental and nutritional conditions for chlamyospore formation in *Phytophthora* species or even isolates might be different. In the present work only 40% of the isolates of *P. parsiana* produced chlamyospore on CA but failed on other media. Infected pistachio and almond roots were better substrate for chlamyospore formation but only 60% of the isolates were capable of producing chlamyospores in root tissue. Cleared V-8 juice broth is a suitable substrate for sporulation of *Phytophthora* species (Ribeiro 1978) but our isolate failed to produce chlamyospores even after twelve months incubation. Chlamyospore formation in *P. cinnamomi* in concentrated CV-8 broth was delayed but quickly produced in diluted CV8 juice (Mircetich and Zentmyer 1967).

Nutrition has great impact on chlamyospore formation in *Phytophthora* species. Chlamyospores of *P. cinnamomi* were not produced on fiber glass covered with mycelium of

the pathogen buried in soil but formed in colonized avocado roots in soil. In the present work chlamyospores of *P. parsiana* produced only on CA and roots of susceptible hosts. The nutritional effect of carrot, potato on growth of the pathogen is not significant and it is speculated that the general nutrients may not be as important as other factors. The triggering factor in carrot juice to induce chlamyospore formation by *P. parsiana* is not known. Carrot root tissue has been shown also to contain some metabolites which inhibit the development of fungi with antifungal activities affecting inhibition of chlamyospore germination in *Mycocentrospora aceriana* (Davies and Lewin 1981). *P. parsiana* isolates did not respond to CV-8 broth. Production of chlamyospores has been reported to be favored by rich nutrient media such as clarified V-8 juice diluted with an equal volume of distilled water (Mitchell and Kanwejer-Mitchell 1992). The carbon nitrogen ratio of the medium and the addition of sterols may strongly influence chlamyospore production (Ribeiro 1978). Colonized roots of pistachio and almond were better substrate for chlamyospore formation by *P. parsiana* than CA medium since more isolates tend to produce chlamyospore in roots than in culture medium. This signifies the importance of natural substrates in intact plant than in culture medium. If proper environmental and nutritional conditions are present most *Phytophthora* species might be able to produce chlamyospores.

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